IN THE SPECIFICATION

Please amend paragraph [0001] – [0067] to include paragraph numbers and as follows:

Field of the invention[[.]]

[0001] The invention relates to ~~the use of~~ methods of using at least one active substance for the prophylaxis ~~and/~~or therapy of a viral disease, wherein at least one active substance inhibits at least one cellular component such that [[a]] virus multiplication is inhibited. The present invention further relates to the combination of at least one such active substance with at least one further different antivirally acting substance for the prophylaxis and/or therapy of at least one viral disease.

Background of the invention and prior art[[.]]

[0002] Infections by RNA or DNA viruses are a substantial threat ~~for~~ to the health of ~~man an animal~~ humans and animals. To the RNA viruses belong the negative-strand-RNA viruses, such as for ~~instance~~ example, influenza viruses or the Borna disease virus. Infections by influenza viruses ~~still belong to the big~~ is the source of large-scale epidemics ~~of mankind~~ and cause ~~year for year~~ a large number of fatalities on an annual basis. They are an immense cost factor ~~for~~ in the economy, for instance by ~~inability to work because of~~ causing lost work days due to illness. Of substantial economic importance are also infections caused by the Borna disease virus (BDV), in particular ~~attacking~~ those that attack horses and sheep, which ~~was however already~~ have been isolated in man, too, and which ~~was~~ have been connected ~~here~~ with neurological diseases.

[0003] The problem of controlling in particular RNA viruses is the adaptability of the viruses caused by a high fault rate of the viral polymerases~~, thus~~. Thus the preparation of suitable vaccines as well as the design of antiviral substances ~~being~~ has been very difficult.

[0004] Furthermore, it has been found that the application of antiviral substances immediately directed against the functions of the virus, ~~initially at the beginning of the therapy have a fair~~ has a relatively fair antiviral effect at the early stage of therapy, but will lead very quickly to the ~~selection~~ generation of resistant variants~~, because of~~ by mutation. An example is the anti-influenza drug amantadine and its derivatives, which is or are directed against a transmembrane

protein of the virus. Within a short time after application, resistant variants of the virus are generated.

[0005] Other examples are the new therapeutic agents for influenza reactions that inhibit ~~inhibiting~~ the influenza-viral surface protein, neuraminidase. Hereto belongs, for instance, Relanza. In patients, Relanza-resistant variants have already been found (Gubareva et al., J Infect Dis 178, 1257-1262, 1998). The hopes placed on this therapeutic agent thus could not be fulfilled.

[0006] Due to their in most cases very small genomes and therefore limited coding capacity for replication-necessary functions, all viruses have to rely to a ~~strong~~ large extent on functions of their host cells. By exerting influence on such cellular functions ~~being~~ necessary for ~~the~~ viral replication, it is possible to negatively affect the virus replication in the infected cell. Then, there is no possibility for the virus to replace the missing cellular function by adaptation, in particular by mutation, in order to avoid the selection pressure. This could already be shown ~~for~~ in the example of the influenza A virus with relatively unspecific inhibiting substances against cellular kinases and methyltransferases (Scholtissek and Müller, Arch Virol 119, 111-118, 1991).

[0007] It is known that cells have a multitude of signal transduction pathways, by means of which signals acting on the cell are transmitted to the cell nucleus. Thereby the cell is able to react to outside stimuli with cell proliferation, cell activation, differentiation or controlled cell death.

[0008] These signal transduction pathways have in common ~~that they include~~ at least one kinase, which activates by phosphorylation at least one protein ~~thereafter transducing~~ that transduces the signal.

[0009] ~~Observing~~ By observing the cellular processes induced ~~after~~ as a result of virus infections, it can be found that a multitude of DNA and RNA viruses activate in the infected host cell preferably by a defined signal transduction pathway, the so-called Raf/MEK/ERK kinase signal transduction pathway. This signal transduction pathway is one of the most important signal transduction pathways in a cell and plays a substantial role in proliferation and differentiation processes (Cohen, Trends in Cell Biol 7, 353-361, 1997; Robinson and Cobb, Curr. Opin. Cell Biol 9, 180-186, 1997; Treismann, Curr. Opin. Cell Biol 8, 205-215, 1996).

[0010] Newer data show that the inhibition of the Ras-Raf-MEK-ERK signal transduction pathway by active substances, which selectively inhibit one or several of kinases involved in this signal transduction pathway, for instance the MEK and/or the SEK, the intracellular multiplication of intranuclearly replicating negative-strand RNA viruses, for in¬stance of influenza A virus and the Borna disease virus (BDV) (PCT/DE 01/01292; PCT/DE 02/02810).

[0011] It is known that viruses can inhibit ~~the~~ apoptosis of the infected cell. This could, for instance, be detected for influenza viruses ~~in vitro and in vivo~~ *in vitro* and *in vivo* (Fesq et al., 1994; Hinshaw et al., 1994; Mori et al., 1995; Takizawa et al.; 1993). ~~It could not fully be clarified,~~ However, it is not yet clear which virus protein acts proapoptotically ~~therein, possibly~~ and whether the apoptosis of the host cell is possibly induced by the generation of interferon (Balachandran et al., 2000) or by proapoptotic virus proteins such as PB1-F2 (Chen et al., 2001).

[0012] ~~It is not clear, which~~ The influence ~~the~~ of virus-induced apoptosis ~~has~~ on ~~the~~ virus multiplication is not as yet clear. ~~There are assumptions~~ One hypothesis is that the release of proapoptotic virus proteins may lead lymphocytes into ~~the~~ apoptosis, and that thereby a reduced immune defense against the virus-infected cells and a promotion of the virus multiplication may result (van Campen et al., 1989; Tumpey et al., 2000). ~~Other assumptions are~~ Another hypothesis is that ~~the~~ apoptosis of the host cell ~~increases by the phagocytosis intensified thereby the~~ is increased by an intensified phagocyctotic immune reaction against the virus (Watanabe et al., 2002). On the other hand, it is known that an overexpression of the antiapoptotically acting Bcl-2 inhibits ~~the~~ virus multiplication (Hinshaw et al., 1994; Olsen et al., 1996). ~~In contrast thereto are findings~~ However, there are contrasting findings that indicate that the inhibition of ~~the~~ virus induced apoptosis by a caspase inhibitor ~~did~~ do not have any influence on the synthesis of virus proteins (Takizawa et al., 1999).

[0013] ~~The~~ Apart from viruses, apoptosis of a cell may be induced, ~~besides by viruses, also by different~~ by other proapoptotic mechanisms and proteins. ~~It is common to these different~~ These other mechanisms and proteins ~~that they~~ commonly activate a proteolytic cellular cascade series of cysteinyl proteases, so-called caspases. The initially activated caspases such as caspase-8 and caspase-9 activate ~~therein~~ the effector cascades such as ~~the~~ caspases-3 and 6. These in turn cleave a series of cellular substrates and cause thereby the apoptosis of the respective cell (surveys in Cohen, 1997; Thornberry and Lazebnik, 1998).

~~Technical object of the invention.~~ Summary of the invention

[0014] It is the object of the invention to provide active substances for pharmaceutical compositions, which show improved antiviral effects, and a test system for identifying such active substances.

~~Findings the invention is based on.~~

~~Surprisingly it has been found that i) for multiplication, influenza viruses need the cellular caspases, in particular caspase-3, and that in cells without caspase-3, the virus-genome ribonucleic protein complexes cannot diffuse through the pores of the nucleus membrane into the cytoplasm, but remain in the nucleus, ii) the inhibition of at least one cellular caspase, in particular the inhibition of the caspase-3 will lead to a distinct inhibition of the multiplication of negative-strand RNA-viruses, in particular of influenza-viruses, and iii) the combination of an inhibitor for a caspase, in particular caspase-3, with another antivirally effective substance, for instance with an inhibitor for a cellular kinase, has a synergistic effect on the inhibition of the virus multiplication.~~

~~The surprising effects of caspase inhibitors on the multiplication of viruses, in particular of negative-strand RNA-viruses, for instance of influenza viruses, is made clear by that this inhibition of the virus multiplication by the inhibition of the caspase is not connected with an inhibition of the synthesis of early or late virus proteins (for instance NP or NS1 (early) still from matrix proteins (M1, late)) and was still observed, when the caspase inhibitor was added only 4 h after the infection.~~

~~Basics of the invention and embodiments.~~ Detailed Description Of The Preferred Embodiments

[0015] For achieving the technical object, the invention ~~therefore~~ teaches ~~the subject matters of the patent claims, in particular~~ i) the use of at least one active substance, which reduces the amount or activity of a cellular caspase, in particular caspase-3, for the prophylaxis and/or therapy of a viral disease, in particular of a viral disease caused by negative-strand RNA viruses, for instance by an influenza virus, ii) the combination of at least one active substance, which

reduces the amount or activity of a cellular caspase, in particular caspase-3, with another antiviral active substance and the use of said combination for the prophylaxis and/or therapy of a viral disease, in particular of a viral disease caused by negative-strand RNA viruses, for instance by an influenza virus, iii) a test system for finding an active substance according to the invention, wherein said test system comprises: 1) a cellular caspase, preferably caspase-3, which is brought into contact with a test substance, and it is measured, whether the protease activity of the caspase ~~reduces~~ is reduced by the test substance, 2) a cell, which is brought into contact with a test substance, and it is measured, whether the amount or activity of a cellular caspase, preferably caspase-3, is reduced by the test substance, 3) a cell, which is infected with a virus, preferably with a negative-strand RNA virus, for instance an influenza virus, and to which thereafter a test substance is added, which is capable ~~to reduce~~ of reducing the amount or the activity of ~~the~~ cellular caspase, in particular caspase-3~~, and it is measured, whether this~~ and the test substance~~'s can inhibit~~ inhibition of the multiplication of the viruses in said cell is measured.

[0016] Surprisingly it has been found that i) for multiplication, influenza viruses need the cellular caspases, in particular caspase-3, and that in cells without caspase-3, the virus-genome ribonucleic protein complexes cannot diffuse through the pores of the nucleus membrane into the cytoplasm, but rather, remain in the nucleus, ii) the inhibition of at least one cellular caspase, in particular the inhibition of the caspase-3 will lead to a distinct inhibition of the multiplication of negative-strand RNA viruses, in particular of influenza viruses, and iii) the combination of an inhibitor for a caspase, in particular caspase-3, with another antivirally effective substance, for instance with an inhibitor for a cellular kinase, has a synergistic effect on the inhibition of virus multiplication.

[0017] The surprising effects of caspase inhibitors on the multiplication of viruses, in particular of negative-strand RNA viruses, for instance of influenza viruses, is made clear in that the inhibition of the virus multiplication by the inhibition of the caspase is not connected with an inhibition of the synthesis of early or late virus proteins (for instance NP or NS1 (early) from matrix proteins (M1, late)). The inhibition effect was still observed even in instances where when the caspase inhibitor was added only 4 hours after infection.

[0018] ~~To the~~ The active substances in the meaning of the present invention ~~belong for instance~~ include for example: peptide and non-peptide inhibitors of the cellular caspase-3, such as Z-DEVD-FMK, Ac-DEVD-CHO, Ac-DMQD-CHO, Z-D(OMe)E(OMe)VD(OMe)-FMK, Z-D(OMe)QMD(OMe)-FMK (all above from Alexis Biochemicals), inhibitors of cellular caspases, which can activate caspase-3, such as peptide and non-peptide inhibitors of the caspase-9, such as Z-LE(OMe)HD(OMe)-FMK, Z-LEHD-FMK, Ac-LEHD-CHO (all from Alexis Biochemicals), peptide and non-peptide inhibitors of the caspase-8, such as Z-LE(OMe)TD(OMe)-FMK, Ac-ESMD-CHO, Ac-IETD-CHO, Z-IETD-FMK (all from Alexis Biochemicals), peptide and non-peptide inhibitors of the caspase-10, such as Ac-AEVD-CHO, Z-AEVD-FMK (both from Alexis Biochemicals), peptide and non-peptide inhibitors of other caspases or granzyme B and pan-caspase inhibitors, such as Z-VAD-FMK, Z-VAD-(OMe)-FMK (both from Alexis Biochemicals), Ac-YVAD-CHO, Z-YVAD-FMK, Z-VDVAD-FMK, Ac-LEVD-CHO (all from Calbiochem), dominant negative mutants of a cellular caspase, in particular of the caspase-3, antisense-oligonucleotides, which specifically accumulate at the DNA sequence or m-RNA sequence coding for a cellular caspase and inhibit the transcription or translation thereof, dsRNA oligonucleotides, which are suitable for the specific degradation of the mRNA's of a cellular caspase by the RNAi technology according to the methods as described by Tuschl et al. (Genes Dev 13:3191-3197, 1999) and Zamore et al. (Cell 101:25-33, 2000), antibodies or antibody fragments specific for a cellular caspase, or fusion proteins containing at least one antibody fragment, for ~~instance~~ example a Fv fragment, which inhibit the protease activity of at least one caspase, inhibitors, which indirectly inhibit the expression or the activation of cellular caspases, in particular caspase-3, ~~expression of~~ or expressed proteins, which inhibitingly act on caspases, for ~~instance~~ example the cellular inhibitors of apoptosis proteins cIAP1, cIAP2, the X-linked inhibitor of apoptosis protein XIAP, the antiapoptotic protein Bcl-2 or the baculoviral protein p35.

[0019] Preferably, ~~the use of~~ at least one active substance according to the invention ~~occurs at the occasion of a~~ is used to prevent or treat a viral disease, which is caused by RNA or DNA viruses, preferably negative-strand RNA viruses, for instance influenza viruses, or Borna viruses.

[0020] Another embodiment of the present invention relates to a combination preparation for the prophylaxis ~~and/~~or therapy of at least one viral disease, containing at least two antiviral active

substances, wherein at least one active substance inhibits a cellular caspase, preferably the caspase-3, and at least one further antiviral active substance.

[0021] ~~To further~~ The antiviral active substances ~~belong for instance~~ include for example, 1-adamantanamine (amantadine), rimantadine, neuraminidase inhibitors such as Relenza, synthetic nucleoside analogs such as 3-deazaadenosine and ribavirin, antivirally acting inhibitors of the cellular kinases, ~~for instance~~ as described, for example, in ~~the~~ PCT patent applications PCT/DE 01/01292 and PCT/DE 02/02810.

[0022] The administration of the combination preparation may take place as a mixture of the active substances. The active substances may, however, also be administered separately at the same place, for instance, intraveneously, ~~i.v.~~, or at separate places, simultaneously or at different times within a period, wherein the substance administered first is still effective, for instance within a period of three days.

[0023] Another embodiment of the present invention relates to a test system for identifying active substances, which inhibit at least one cellular caspase, in particular the caspase-3, such that the multiplication of viruses, in particular of negative-strand RNA viruses, for instance of influenza viruses, comprising [[a.]] a) at least one cell infectable with at least one virus and comprising either at least one caspase, in particular caspase-3, and at least one virus infecting the cells, or [[b.]] b) at least one cell infectable with at least one virus and comprising at least one caspase, in particular caspase-3.

[0024] Cells in the meaning of the present invention are cells from different organs and tissues, for ~~instance~~ example, cells of the blood and lymphatic vessels, and cells~~, which cover~~ covering the body cavities. Equally comprised are cell cultures, in particular ~~such,~~ those which can be acquired from cell banks, such as American Type Culture Collection ("ATCC"), in particular permissive, eukaryotic cell cultures, for ~~instance~~ example A549 (*homo sapiens*) B82, NIH, 3T3, L929, all from *Mus musculus*, BHK from *Cricetus cricetus*, CHO from *Cricetulus griseus*, MDCK from *Canis famliliaris*, vero, COS-1 and COS-7, all from *Cercopithecus aethiops*, and primary embryo fibroblasts from *Gallus gallus* (CEF cells).

[0025] For instance, ~~in~~ the test system according to the invention for identifying active substances, ~~it is tested by~~ includes the addition of substances, preferably in concentrations of 0.001 µmole to 100 µmoles, and viruses in a particle number, which can infect the selected cell,

and determining whether a substance is capable ~~to inhibit the~~ of inhibiting virus multiplication[[,]] without damaging the cell.

[0026] Preferably, the virus used in the test system according to the invention is an RNA or DNA virus, preferably an influenza virus.

[0027] In a preferred embodiment, the cell of the test system according to the invention contains at least one overexpressed caspase, in particular caspase-3, in particular by the introduction of one gene or several genes influencing ~~the~~ caspase. By this overexpression, substances are detected, which can strongly inhibit caspases as well as intracellularly reach a high concentration for the inhibition of the overexpressed caspases.

[0028] For control purposes, the expression ~~for~~ of at least one caspase, preferably ~~the~~ caspase-3, in a cell of a test system according to the invention is inhibited, for instance a) by the introduction of an antisense DNA or an antisense RNA, or b) by the introduction of at least one gene coding for at least one dominant-negative mutant of at least one caspase.

[0029] Another embodiment of the present invention relates to a method for identifying at least one active substance according to the invention for the prophylaxis and/or therapy of viral diseases, which inhibits the multiplication of viruses during viral diseases, comprising the following steps: [[a.]] a) bringing at least one test system according to the invention into contact with at least one potential active substance, and [[b.]] b) determining the effects on ~~the~~ virus multiplication.

[0030] "Bringing into contact" in the meaning of the present invention may, for instance, occur by the addition of the active substances into the culture medium of a cell culture or by local or systemic administration of the active substances into an organism.

[0031] "Bringing into contact" in the meaning of the present invention also comprises ~~the~~ prior art methods, which permit the introduction of substances into intact cells, for instance, infection, transduction, transfection and/or transformation and other methods known to ~~the man~~ one skilled in the art. These methods are in particular preferred~~, if the substance comprises~~ for substances comprising viruses, naked nucleic acids, for ~~instance~~ example antisense DNA and/or antisense RNA, viroids, virosomes and/or liposomes~~, and virosomes~~. Virosomes and liposomes are also suitable ~~to bring~~ for bringing further active substances into the cell[[,]] besides a nucleic acid molecule.

[0032] The determination of the effects on ~~the~~ virus multiplication occurs, for instance, by plaque assays or comparison of the virus titer of [[-]]infected ~~or~~ and non-infected cells.

[0033] Another preferred embodiment of the present invention relates to a method for preparing a drug for the prophylaxis and/or therapy of at least one viral disease, which substantially inhibits ~~or inhibit~~ the multiplication of viruses, comprising the following steps: [[a.]] a) performing a test system according to the invention, and [[b.]] b) reacting the active substance(s) with at least one auxiliary and/or additional substance.

[0034] Preferably, the active substance according to the invention is processed for ~~the~~ local or systemic administration to an organism by using methods known to ~~the man~~ one skilled in the art and auxiliary and/or additional substances ~~to~~ for a drug.

[0035] Suitable auxiliary and/or additional substances, for example, substances ~~which serve for instance~~ for stabilizing or preserving the drug or diagnostic agent, are generally known to ~~the man~~ one skilled in the art (see e.g. Sucker H et al. (1991) Pharmazeutische Technologie, 2nd edition, Georg Thieme Verlag, Stuttgart). Examples ~~for~~ of such auxiliary and/or additional substances are physiological common salt solutions, Ringer's dextrose, dextrose, Ringer's lactate, demineralized water, stabilizers, antioxidants, complex-forming agents, antimicrobial compounds, proteinase inhibitors and/or inert gases.

[0036] The local administration may, for instance, be made on the skin, on the mucous membrane, into a body cavity, into an organ, into a joint or into the connective or supporting tissue, by nasal administration or by inhalation. The systemic administration preferably occurs into the blood circulation, into the peritoneal cavity or into the abdominal cavity.

[0037] The drug preparation comprising the active substance according to the invention depends on the type of active substance and the ~~way~~ method of administration and may, for instance, be a solution, a suspension, an ointment, a powder, a spray, or another inhalation preparation. Preferably, nucleotide sequences are inserted by methods well known to ~~the man~~ one skilled in the art into a viral vector or a plasmid and reacted with auxiliary substances for ~~the~~ cell transfection. ~~To these~~ These auxiliary substances ~~belong for instance~~ include, for example, cationic polymers or cationic lipids. Antisense oligonucleotides are derivatized by methods familiar to ~~the man~~ one skilled in the art, in order to protect them from enzymatic degradation by DNAses or RNAses.

[0038] The active substance according to the invention may be present in the form of a salt, ester, amide or as a ~~pre-stage~~ precursor, and preferably only such modifications of the active substance are used, which do not cause ~~any excessive~~ severe toxicity, irritations or allergic reactions ~~of~~ in the patient.

[0039] The active substance is mixed under sterile conditions with a physiologically acceptable carrier substance and potential preservation agents, buffers or driving agents, depending on the application. Such carrier substances for the drug preparations are familiar to ~~the man skilled~~ one of ordinary skill in the art.

[0040] Preferably, the active substance according to the invention is administered ~~in~~ as a one-time dose, ~~in particular~~ preferably in several doses, and the individual doses do not exceed the maximum tolerable dose (MTD) of the respective active substance for ~~man~~ humans. Preferably, a dose is selected, ~~which~~ that is half the MTD.

[0041] According to the present invention, the administration may take place either locally or systemically, only on one day or daily over several days or at every second or third day over several weeks, until a therapeutic effect is visible.

[0042] In the following, the invention will be explained in more detail with reference to examples representing embodiments only.

Example 1: virus multiplication in wildtype and in caspase-3-deficient cells[[.]]

[0043] In order to analyze whether caspases, in particular caspase-3, play an important role in ~~the~~ influenza virus multiplication, the activity and expression of the protease(s) was inhibited in four different ways: a) by the addition of a cell-permeable inhibitor (Z-DEVD-FMK), which preferably inhibits the caspase-3 activity, besides other caspases, b) by expression of an inhibitory protein of caspases, XIAP (X-linked inhibitor of apoptosis) (Devereaux et al., Nature, 388, 300-304, 1997), which inhibits caspase-3, among others, c) by stable transfection of a vector, which forms a siRNA against the mRNA of caspase-3, d) by investigation of a cell line (MCF-7), which is caspase-3-deficient (Jänicke et al., J Biol Chem, 273, 9357-9360) and which was complemented by a transient transfection with procaspase-3.

[0044] ~~With regard to~~ Regarding a), ~~the following was made.~~ MDCK cells were infected with the influenza A virus strain Bratislava/79 (fowl plague virus, FPV) with a multiplicity of infection of

1 (M.O.I. = 1) in the absence and in the presence of increasing amounts of the caspase-inhibitor Z-DEVD-FMK (2, 4, 20, 40 µM, Alexis Biochemicals), which preferably inhibits caspase-3. The concentration of DMSO corresponding to the highest inhibitor amount (2 %) served as a solvent control. As another control, the inactive inhibitor, analog Z-FA-FMK, was used in a concentration of 40 µM. After 24 hours, the cell supernatants were investigated with conventional methods (plaque titration) ~~with regard to~~ to determine the amount of newly formed viruses. In parallel thereto, the effect of the caspase-inhibitor was analyzed by ~~the measure of~~ by measuring the cleavage of the cellular caspase-substrate PARP (poly-ADP ribose polymerase), which is cleaved for instance by caspase-3 (Tewari et al., Cell, 81, 801-809, 1995), in the Western blot of cell lysates. The effects of the inhibitor on the expression of early or late viral proteins (NS1, NP, M1) were also investigated in the Western blot. In a variant of the experiment, the inhibitor, DEVD-FMK, was added in a concentration of 40 µM, and was washed away ~~already after 2 h after the~~ 2 hours after infection and replaced by fresh medium, or was added 4 hours ~~only~~ after ~~the~~ infection. In another modification of the experiment, the broadband caspase-inhibitor, Z-VAD-FMK, was used for comparison ~~reasons~~ purposes in analogous concentrations in A549, MDCK as well as in vero cells.

[0045] ~~With regard to~~ Regarding b), ~~the following was made.~~ MDCK cells were transfected with a vector plasmid or with plasmids, which express XIAP or procaspase-3. The transfection was performed with the transfection reagent Lipofectamine 2000 (Life Technologies) according to standard methods (Ludwig et al., J Biol Chem, 276, 10990-10998, 2001). The transfection efficiencies were approx. 60 %. 24 hours after ~~the~~ transfection, the infection with the influenza A virus strain fowl plague virus (FPV) occurred with a multiplicity of infection of 1 (M.O.I. = 1). Another 24 hours after ~~the~~ infection, the titers of the newly formed viruses in the cell culture supernatant were investigated in standard plaque assays for MDCK cells. The successful expression of the transiently expressed proteins was verified in the Western blot.

[0046] ~~With regard to~~ Regarding c), ~~the following was made. The~~ the lung epithelial cell line, A549, was transfected ~~by~~ using standard methods (Lipofectamine 2000 (Life Technologies); Ludwig et al., J Biol Chem, 276, 10990-10998, 2001) with the vector, pSUPER, which leads to the generation of small interfering dsRNA fragments in the cell (siRNA) (Brummelkamp et al., Science, 296, 550-553, 2002). ~~As inserts were used the~~ The following target sequences of the

caspase-3 were used as inserts (gene bank association No. NM004346): TGACATCTCGGTCTGGTAC (nt 417-435), CTGGACTGTGGCATTGAGA (734-755) and TAC¬CAGTGGAGGCCGACTT (795-813) (clones #113, #252 and #311). ~~In another clone (#313)~~ An insertion was identified in another clone (#313) ~~an insertion~~. Thus this clone served as a negative control. The constructs were transfected together with the vector pCAGI-puro, in order to make the cells selectable with the antibiotic puromycin. 24 hours after ~~the~~ transfection, the cells were washed and then incubated with medium, which contained 1 µg/ml puromycin. 24 hours later, the cells were ~~intensely~~ thoroughly washed with PBS, and new antibiotic-containing medium was added. This procedure was then repeated for 7 days in the presence of 0.6 µg/ml puromycin. After 7 days, the different cells were investigated for the expression of ~~the~~ caspase-3. Also after 7 days, the infection of the different cell lines with influenza A virus strain fowl plague virus (FPV) occurred with a multiplicity of infection of 1 (M.O.I. = 1). Another 24 hours after ~~the~~ infection, the titers of the newly formed viruses in the cell culture supernatant were tested in standard plaque assays for MDCK cells.

[0047] ~~With regard to~~ Regarding d), ~~the following was made. The~~ the caspase-3-deficient breast carcinoma cell line MCF-7 was transfected with a vector plasmid or a plasmid, which expresses procaspase-3. The transfection was performed with the transfection reagent Lipofectamine 2000 (Life Technologies) according to standard methods (Ludwig et al., J Biol Chem, 276, 10990-10998, 2001). The transfection efficiencies were approx. 50 %. 24 hours after ~~the~~ transfection, the infection with the influenza A virus strain fowl plague virus (FPV) occurred with a multiplicity of infection of 1 (M.O.I. = 1). Another 24 hours after ~~the~~ infection, the titers of the newly formed viruses in the cell culture supernatant were investigated in standard plaque assays for MDCK cells. The successful expression of procaspase-3 was verified in the Western blot.

The following results were obtained:

[0048] a) In a dose-dependent manner, the ~~The~~ caspase-3-inhibitor, Z-DEVD-FMK, ~~led in dependence on the dose to the~~ caused a reduction ~~of~~ in the influenza virus titers after 24 hours up to approx. 60 % ~~inhibition~~ at a concentration of 40 µM. This inhibition precisely correlated with the obtained caspase inhibition, measured by the cleavage of the caspase substrate PARP. This shows that the level of the activity of cellular caspases, in particular of caspase-3, directly

correlates with the efficiency of ~~the~~ virus multiplication, and that caspase inhibitors can be used for the inhibition of influenza multiplication. The same efficiency of the inhibition of the virus multiplication was obtained with a pan-caspase inhibitor, Z-VAD-FMK, in A549 cells as well as in MDCK and vero cells, whereas an inactive inhibitor analog, Z-FA-FMK, did not show any effect. In addition, it was observed that in spite of the inhibiting effect on ~~the~~ virus multiplication, Z-DEVD-FMK had no significant effect on ~~the~~ virus protein expression. This ~~is a piece of evidence for~~ demonstrates that caspase activity ~~is needed~~ occurs relatively ~~lately~~ late in the virus replication cycle. This is supported by the finding that the inhibitor continued to effectively inhibit~~ing the~~ virus multiplication~~, if it is~~ when added only 4 hours after ~~the~~ infection, ~~i.e.~~ *i.e.* at late ~~times~~ phases of the replication cycle. The presence of Z-DEVD-FMK in the first two hours after the infection had no significant effects if removed thereafter.

[0049] b) The expression of the caspase-inhibitory protein, XIAP, led to a reduction of the influenza virus titer of approx. 50 % after 24 hours. This reduction substantially correlated with the obtained inhibition of the caspase activity, measured by the reduced cleavage of the protein PARP, which also was approx. 40 - 50 % of the efficiency of the original activity. On the other hand, an increased virus multiplication ~~could be~~ was observed in the transfected cells by expression of the procaspase-3. This once again verifies that the level of the caspase-3 activity in cells directly correlates with the efficiency of ~~the~~ influenza virus replication.

[0050] c) Western blot experiments showed that a stable expression of different siRNA segments in A549 cells led to a more or less strong reduction ~~of~~ in the protein levels of caspase-3 in the different cell lines, whereas the expression of a control siRNA segment did not show any effects. According to the degree of reduction of the protein amount, gradual effects on the influenza virus replication in the different lines were obtained, and the strongest inhibiting siRNA segment (#113) led to an approx. 10-fold reduction of the virus titers. The corresponding control siRNA segment (#313) ~~correspondingly not had any~~ did not have an effect on ~~the~~ virus multiplication. It should be noted ~~here~~ that the strong expression inhibition of caspase-3 led to an increased expression and activity of the caspase-7 in the cell line, #113. This effect, which can be understood as a compensation reaction of the cell, could, however, not eliminate the defects of ~~the~~ virus multiplication.

[0051] d) The infection of wildtype or vector-transfected MCF-7 cells led to very few titers ~~only~~ of descendant viruses, and this indicates that the replication efficiency of influenza A viruses in these caspase-3-deficient cells is very ~~small~~ low. If, however, procaspase-3 was introduced by transient transfection into these cells, a 30-fold increase ~~of~~ in the titers of descendant viruses was observed, ~~another piece of~~ providing additional evidence of the importance of caspases, in particular caspase-3, for ~~an~~ efficient influenza virus multiplication.
[0052] In ~~total~~ summary, these results ~~have as a consequence~~ prove that the degree of expression and activity of caspases, in particular caspase-3, directly correlate[[s]] with the efficiency of the influenza virus replication. ~~Correspondingly~~ Thus, caspases, in particular caspase-3, are target points for an anti-influenza virus prophylaxis or therapy.

Example 2: Mechanism of the inhibition of ~~the~~ virus multiplication by a caspase inhibitor[[.]]
[0053] Western blot analyses of cell lysates of caspase inhibitor-treated influenza virus-infected cells showed that in spite of efficient inhibition of ~~the~~ virus multiplication, there was no effect on ~~the~~ viral protein synthesis, and thus a late step of the replication cycle, when the viral protein synthesis is substantially accomplished, seems to be affected by caspase activity (also see results for a)). This is supported by findings~~, which~~ that show that caspase inhibitors still have an efficient inhibition ~~of the~~ on virus multiplication, if they are added 4 hours ~~only~~ after ~~the~~ infection, ~~i.e.~~ *i.e.* at a late ~~time~~ phase in the infection cycle, whereas the presence of the substances in the first two hours of the infection did hot have any effect if removed thereafter. An essential step late in the infection cycle of influenza viruses is the export of newly formed viral RNA in the form of ribonucleic protein complexes (RNP's) from the cell nucleus of the infected cell. Recently ~~could be shown that the~~ it was shown that caspase activity in ~~the~~ cells leads to an expansion of the nucleus pores, which permits a free diffusion of large proteins or protein complexes between the cell nucleus and the cytoplasm (Falerio and Lazebnik, J. Cell Biol, 151, 951-959, 2000). In order to analyze[[,]] whether caspase activity ~~regulatingly influences~~ regulates the export of viral proteins or RNP's from the cell nucleus, and whether this happens by free diffusion of the proteins, the following experimental batches were performed: a) wildtype A549 cells and A549 cells, which carry a caspase-3 siRNA, were infected and tested in immunofluorescence analyses for the localization of the RNP's, b) wildtype MDCK cells were

treated and infected with the caspase-3 inhibitor Z-DEVD-FMK, and were also tested in immunofluorescence analyses for the localization of the RNP's, c) MDCK cells were transfected with a plasmid for the influenza A virus nucleoprotein (NP), and the localization of the NP was tested in immunofluorescence analyses after apoptosis induction with staurosporine in the presence and in the absence of a caspase inhibitor, d) MDCK cells were transfected for the reconstitution of RNP complexes with plasmids, which code for the NP and the influenza polymerases PB2, PB1 and PA, and with a plasmid, which expresses an influenza virus-specific RNA matrix. The effects on the localization of the RNP complexes were investigated in the presence and in the absence of caspase inhibitors in immunofluorescence analyses.

[0054] ~~With regard to~~ Regarding a), ~~the following was made.~~ A549 cells or A549 cell lines, which carried the siRNA segment #113, were infected with the influenza A virus strain fowl plague virus (FPV) with a multiplicity of infection of 3 (M.O.I. = 3). 5 hours after ~~the~~ infection, the cells were forwarded by conventional methods (Pleschka et al., Nat cell Biol, 3, 301-305, 2001) to the immunofluorescence analysis with a goat anti-NP antiserum (Robert Webster, Memphis, USA) and an anti-goat Texas red-IgG secondary antibody (Dianova). The cell nuclei were stained with DAPI. The visualization occurred by an inverse fluorescence microscope ~~in~~ at a magnification of 40.

[0055] ~~With regard to~~ Regarding b), ~~the following was made.~~ MDCK cells were infected with the influenza A virus strain fowl plague virus (FPV) with a multiplicity of infection of 5 (M.O.I. = 5) in the presence of DMSO (2 %), caspase-3 inhibitor Z-DEVD-FMK (40 µM, Alexis Biochemicals), the inactive inhibitor analog Z-FA-FMK (40 µM, Alexis Biochemicals) or the MEK inhibitor U0126 (50 µM, Taros Coustom Biochemicals). 5 hours after ~~the~~ infection, the cells were forwarded by conventional methods (Pleschka et al., Nat cell Biol, 3, 301-305, 2001) to the immunofluorescence analysis with a goat anti-NP antiserum (Robert Webster, Memphis, USA) and an anti-goat Texas red-IgG secondary antibody (Dianova). The cell nuclei were stained with DAPI, the staining of the cytoskeleton was made with phalloidin-FITC. The visualization occurred by an inverse fluorescence microscope ~~in~~ at a magnification of 40.

[0056] ~~With regard to~~ Regarding c), ~~the following was made.~~ MDCK cells were transfected with plasmids, which code for the influenza A virus proteins PB2, PB1, PA and NP, and with a plasmid, which forms an antisense RNA for the green fluorescent protein accompanied by

influenza virus-specific promoter elements as a matrix for the polymerase complex. It is known that the expression of these plasmids leads to a reconstitution of the RNP complexes, which is shown by the expression of the reporter gene, ~~here~~ in this instance GFP (Pleschka et al., J Virol, 70, 4188-4192, 1996). The transfection was performed with the transfection reagent Lipofectamine 2000 (Life Technologies) according to standard methods (Ludwig et al., J Biol Chem, 276, 10990-10998, 2001). 16 hours after the transfection, the cells were treated for 5 hours with DMSO, staurosporine (1 M) and DMSO, staurosporine (1 M) and Z-DEVD-FMK (40 µM) or staurosporine (1 M) and leptomycin B (2 ng/ml). Then the cells were forwarded by conventional methods (Pleschka et al., Nat cell Biol, 3, 301-305, 2001) to the immunofluorescence analysis with a goat anti-NP antiserum (Robert Webster, Memphis, USA) and an anti-goat Texas red-IgG secondary antibody (Dianova). The cell nuclei were stained with DAPI. The visualization occurred by an inverse fluorescence microscope ~~in~~ at a magnification of 40.

[0057] ~~With regard to~~ Regarding d), ~~the following was made.~~ MDCK cells were transfected with a plasmid, which codes for the influenza A virus NP. The transfection was performed with the transfection reagent Lipofectamine 2000 (Life Technologies) according to standard methods (Ludwig et al., J Biol Chem, 276, 10990-10998, 2001). 16 hours after the transfection, the cells were treated for 5 hours with DMSO, staurosporine (1 M) and DMSO, staurosporine (1 M) and Z-DEVD-FMK (40 µM) or staurosporine (1 M) and U0126 (40 µM). Then the cells were forwarded by conventional methods (Pleschka et al., Nat cell Biol, 3, 301-305, 2001) to the immunofluorescence analysis with a goat anti-NP antiserum (Robert Webster, Memphis, USA) and an anti-goat Texas red-IgG secondary antibody (Dianova). The cell nuclei were stained with DAPI. The visualization occurred by an inverse fluorescence microscope ~~in~~ at a magnification of 40.

The following results were obtained.

[0058] a) ~~The comparison~~ Comparison of the localization of the RNP complexes (~~according to~~ by the detection with an antiserum against the viral nucleoprotein (NP), the main component of the RNP's), showed that the infected A549 cells, ~~which have in dependence from~~ as a function of siRNA, have a strongly reduced expression of caspase-3~~, the~~. The RNP's are held back for 5

hours after ~~the~~ infection in the cell nucleus, whereas in the wildtype A549 cells, for ~~at~~ the same time, the RNP's are already accumulated efficiently in the cytoplasm. This shows that the degree of ~~the~~ caspase-3 expression correlates with the migration efficiency of the RNP's out of the cell nucleus and indicates that this effect is caspase-3-mediated.

[0059] b) ~~The comparison~~ Comparison of the localization of the RNP complexes in infected MDCK cells, which were incubated with solvent or different inhibitors, showed that the migration of the RNP's out of the cell nucleus 5 hours after ~~the~~ infection can efficiently be inhibited by the caspase inhibitor, Z-DEVD, as well as by the MEK inhibitor, U0126, not however by the inactive caspase inhibitor analog, Z-FA-FMK. This ~~is another piece of evidence~~ demonstrates that caspases, in particular caspase-3, mediate the efficient export of RNP's out of the cell nucleus.

[0060] c) After transient expression in unstimulated cells, the influenza virus NP showed [[a]] nuclear localization. If, however, in these cells the caspase activity was induced by the addition of the apoptosis inductor, staurosporine, the nucleoprotein ~~could be found~~ was found to be distributed over the complete cell. This "bleeding" out of the cell nucleus ~~could~~ can be prevented by the addition of the caspase-3 inhibitor Z-DEVD, not however by an inhibitor of the active nucleus export, leptomycin B. This indicates that the caspase activity mediates the free diffusion of large proteins presumably by a proteolytic expansion of the nucleus pores and thus promotes the migration of the NP into the cytoplasm.

[0061] d) After the transient expression of the viral proteins PB2, PB1, PA and NP in MDCK cells, which, starting from a plasmid, ~~in addition~~ additionally express an RNA matrix with influenza virus-specific promoter regions, which accompany a GFP reporter gene, cells with a green staining were found in the culture dish~~, which~~. This indicates that ~~in these cells~~ intact RNP complexes were formed in these cells. GFP as well as the RNP complexes were found in the nucleus of the unstimulated cells ~~in the nucleus. If however~~. However, when ~~in these cells the~~ caspase activity was induced in these cells by the addition of the apoptosis inductor, staurosporine, the GFP as well as the RNP complexes ~~could be found again~~ were found once again to be distributed over the complete cell. ~~Correspondingly, this~~ This "bleeding" out of the cell nucleus ~~could~~ can be prevented by the addition of the caspase-3 inhibitor, Z-DEVD, not however by an inhibitor of the active nucleus export, U0126. This indicates that the caspase

activity mediates the free diffusion of very large protein complexes presumably by a proteolytic expansion of the nucleus pores and thus promotes the migration of the RNP's into the cytoplasm. Further, it is interesting that the respective cells, verified by the ~~inhibitability with~~ inhibiting response observed using Z-DEVD-FMK, ~~have~~ possess caspase activity~~, however otherwise~~. However, no other morphologic signs of apoptotic cells, such as membrane blebbing or condensed nuclei, was observed. This shows that initial events of the apoptosis induction, such as early caspase activity, are already sufficient for mediating the better nucleus export of the protein complexes. Full execution of the apoptotic program is not necessary ~~or would~~ and may even be counterproductive.

Example 3: The synergistic effect of a caspase inhibitor and a kinase inhibitor in the inhibition of ~~the~~ virus multiplication[[.]]

[0062] It is known that the export of influenza virus RNP's is mediated at least in part by ~~the~~ active nucleus export (O'Neill et al., EMBO J, 17, 288-296, 1998), and can correspondingly be inhibited by inhibitors of the active nucleus export machinery such as leptomycin B. It is also known that the RNP export can be inhibited in the late phases of ~~the~~ replication by inhibition of the Raf/MEK/ERK kinase cascade, for instance by the MEK inhibitor U0126, ~~and here this is interfering~~ which interferes with an active export mechanism. Surprisingly, it has been found in conjunction with the invention that the nucleus export of influenza virus RNP's can alternatively also be inhibited by caspase inhibitors, ~~and here this is mainly blocking~~ which in the present instance, would mainly be the blocking of a passive process.

[0063] Now it was intended to find out, whether a) the caspase-activating signal pathway and the Raf/MEK/ERK cascade influence each other, and whether b) by the inhibition of the active export by U0126 and the simultaneous inhibition of the increased passive diffusion by caspase inhibitors, ~~i.e.~~ *i.e.* so to speak blocking of two alternative export mechanisms, a synergistic inhibition effect on the influenza virus replication can be obtained.

[0064] ~~With regard to~~ Regarding a), ~~the following was made.~~ A549 cells were infected with the influenza A virus strain fowl plague virus (FPV) with a multiplicity of infection of 1 (M.O.I. = 1) in the presence of DMSO (2 %), the caspase-3 inhibitor Z-DEVD-FMK (40 µM, Alexis Biochemicals) or the MEK inhibitor U0126 (40 µM, Taros Coustom Biochemicals). 24 hours

after ~~the~~ infection, the cells were lysated, and the lysates were forwarded by conventional methods (Pleschka et al., Nat cell Biol, 3, 301-305, 2001) to an anti-PARP Western blot for the determination of the caspase activity as well as to an ERK immunocomplex kinase assay for the determination of the activity of the Raf/MEK/ERK signal pathway in the infected and treated cells.

[0065] ~~With regard to~~ Regarding b), ~~the following was made.~~ A549 cells and caspase-3-deficient MCF-7 cells were infected with the influenza A virus strain fowl plague virus (FPV) with a multiplicity of infection of 1 (M.O.I. = 1) in presence of DMSO (2 %), the caspase-3 inhibitor Z-DEVD-FMK (40 µM, Alexis Biochemicals) or the MEK inhibitor U0126 (40 µM, Taros Coustom Biochemicals). 9 hours and 24 hours after ~~the~~ infection, the titers of the newly formed viruses in the cell culture supernatant were tested in standard plaque assays for MDCK cells.

The following results were obtained.

[0066] a) The inhibition of caspase-3 in infected cells led to a reduced cleavage of the caspase substrate PARP, not however to a reduced activity of the Raf/MEK/ERK signal pathway, measured by the degree of virus-induced activity of ERK in the immunocomplex kinase assay, which was substantially identical in DMSO and Z-DEVD-FMK-treated cells. Further, the inhibition of MEK by U0126 in concentrations, which efficiently inhibited the virus-induced activity of ERK, did not lead to a modified cleavage of PARP. This indicates that the caspase-3-dependent cascade and the Raf/MEK/ERK signal pathway mediate independently from each other in different processes, which alternatively promote the RNP export and thus make ~~the~~ virus multiplication more efficient.

[0067] b) If, in A549 cells, ~~simultaneously~~ caspases, in particular caspase-3, were inhibited by Z-DEVD-FMK and the Raf/MEK/ERK cascade simultaneously, a synergistic inhibitory effect on the virus replication could be observed after 9 hours as well as after 24 hours. Thereby, the suboptimum inhibitory effect of less than one power of ten by isolatedly used agents could be increased up to the >1 log step by a combined administration. In caspase-3-deficient MCF-7 cells, the Z-DEVD-FMK did not have any effect, as expected, on the virus multiplication. However, the already small titers of descendant viruses from these cells were again reduced by using U0126. These findings showed that in fact, the caspase cascade and the Raf/MEK/ERK

signal pathway regulate two alternative processes for the efficient support of ~~the~~ virus multiplication, and that according to these findings the combined application of caspase inhibitors and MEK inhibitors are ideally suited to inhibit the multiplication of influenza viruses.